PLANT ENZYME AND USE THEREOF

TECHNICAL FIELD

[0001] The present invention relates to use of a plant enzyme gene for transformation. More specifically, the invention relates to use of a previously not described phospholipid acyl hydrolase gene in combination with a gene for an uncommon fatty acid for obtaining transgenic plants comprising both said genes.

BACKGROUND OF THE INVENTION

[0002] There is considerable interest world-wide in producing chemical feedstocks, such as fatty acids, for industrial use from renewable plant resources rather than non-renewable petrochemicals. This concept has broad appeal to manufactures and consumers on the basis of resource conservation and provides significant opportunity to develop new industrial crops for agriculture.

[0003] There is a diverse array of unusual fatty acids in oils from wild plant species and these have been well characterized (see e.g. Badami & Patil, 1981). Many of these acids have industrial potential and this has led to interest in domesticating relevant plant species to enable agricultural production of particular fatty acids.

[0004] Development in genetic engineering technologies combined with greater understanding of the biosynthesis of

unusual fatty acids, now makes it possible to transfer genes coding for key enzymes involved in the synthesis of a particular fatty acid from a wild species into domesticated oilseed crops. In this way individual fatty acids can be produced in high purity and quantities at moderate costs.

PRIOR ART

[0005] Within prior art it is known that plant tissues accumulating triacylplycerols with high amount of medium chain (fatty acids shorter than 16 carbon atoms), hydroxy fatty acids, epoxy fatty acids and acetylenic acids have low concentration of these acids in their membrane lipids (phospholipids). (Stymne et al 1990; Bafor et al., 1990, 1991, 1993; Kohn et al., 1994).

[0006] Furthermore it is known that diacylplycerols is a common precursor for both phospholipids and triacylglycerols in plant tissues accumulating triacylglycerols (see Stymne, 1993a for review). There is also known that CDP-choline choline phosphotransferase in plant tissues accumulating high amounts of medium chain and hydroxy fatty acids in their triacylplycerols do not discriminate against diacylglycerols containing these fatty acids in the synthesis of phosphatidylcholine (Vogel & Browse, 1995).

[0007] Prior art also describes that tissues naturally accumulating triacylplycerols with medium chain fatty acids,

epoxygenated fatty acids and hydroxylated fatty acids have membrane associated acyl hydrolase activities with high specifities towards phospholipids containing the particular uncommon fatty acid this tissue is accumulating, but low activity for common membrane fatty acids (Stymne, 1993, Stahl et al., 1995).

[0008] Furthermore, prior art describes that rape seed genetically engineered to produce dodecanoic (lauric) acid in their seeds have much higher content of that acid in seed phospholipids than two plant species naturally accumulating lauric acids to approximately the same relative level (Wiberg et al, 1995).

[0009] Finally, there exists prior art concerning an anonymous expressed cDNA sequences from young shoots of rice (ID's: D49050, D47724, D47653, D47320) deposited by the Rice genome Research Program in the GenBank.

SUMMARY OF THE INVENTION

[0010] Many of the unusual fatty acids of interest, e.g. medium chain fatty acids, hydroxy fatty acids, epoxy fatty acids and acetylenic fatty acids, have physical properties that are distinctly different from the common plant fatty acids. The present inventors have found that, in plant species naturally accumulating these uncommon fatty acids in their seed oil (triacylplycerols), these acids are absent, or

present in very low amounts, in the membrane (phospho) lipids of the seed. The low concentration of these acids in the membrane lipids is most likely a prerequisite for proper membrane function and thereby for proper cell functions. The idea underlying the invention is that uncommon fatty acids can be made to accumulate to high amounts in seeds of transpenic crops if these uncommon fatty acids are, more or less, excluded from the membrane lipids of the seeds.

[0011] The present invention relates to genetically engineering of oil seeds, oleogeneous yeast and moulds to accommodate high amounts of uncommon fatty acids in their triacylglycerols by introducing genes coding for phospholipid hydrolases, below also called phospholipases, that specifically removes these fatty acids from the membrane lipids of the cell.

[0012] The inventors have identified phospholipase (phospholipase A_2) enzymes responsible for the removal of medium chain fatty acids from phospholipids in plants.

[0013] Thus, in a first aspect the present invention relates to cDNA or genomic DNA coding for a phospholipid acyl hydrolase comprising a nucleotide sequence coding for an amino acid sequence with homology to *Ulmus glabra* phospholipase A_2 as presented in Fig. 7 or amino acid sequences homologous to

those encoded by the rice cDNA clones D49050, D47724, D47653 as presented in Fig. 6 and 7.

[0014] In a second aspect, the invention relates to the use of a plant phospholipid hydrolase gene (cDNA or genomic DNA coding for a phospholipid hydrolase) in combination with a gene for an uncommon fatty acid for obtaining transgenic plants comprising both said genes.

[0015] Preferably, the enzyme encoded by said phospholipid acyl hydrolase gene, or cDNA, is coding for a low molecular weight phospholipase A_2 with distinct acyl specificity for uncommon fatty acids, such as medium chain, long chain (>C_{18}), hydroxy, epoxy and acetylenic acids.

[0016] In a third aspect, the invention relates to transgenic oil accumulating organisms comprising, in their genome, a plant phospholipid hydrolase gene having specificity for a particular uncommon fatty acid and the gene for said uncommon fatty acid.

[0017] Preferably said organisms are selected from the group consisting of oil crops, yeasts, and moulds.

[0018] In a fourth aspect, the invention also relates to oils from such organisms.

DETAILED DESCRIPTION OF THE INVENTION

[0019] Studies by the inventors on the biosynthesis and metabolism of uncommon fatty acids (i.e. medium chain fatty

acids, hydroxy fatty acids, epoxy fatty acids) in different oil seeds (Bafor et al., 1991, 1993; Banas et al., 1993, Stymne, 1993, Stahl et al., 1995), led the inventors to conclude that microsomal phospholipid acyl hydrolases (phospholipases) with specifities towards uncommon acyl groups was, at least in part, responsible for the removal of these acids from the phospholipids in the developing oil seeds.

[0020] It was also shown that the acyl specificities of the phospholipid acyl hydrolases from different plant species were correlated with the type of accumulated fatty acid in the plants.

[0021] Elm (*Ulmus glabra*) seed triacylplycerols are mainly composed of octanoic (8:0) and decanoic (10:0) acids, but these acids are very low in concentrations in the phospholipids of the seeds (Stahl et al., 1995). Membrane fractions (microsomal preparations) from developing Ulmus glabra seeds had high phospholipase A_2 (PLA_2) activity towards phosphatidylcholine with medium chain fatty acids in position sn-2 (octanoic, decanoic and dodecanoic (12:0) acids but very low activity towards phosphatidylcholine with octadeca-9-enoic acid (oleic acid - a common fatty acids) (Stymne, 1993, Stahl et al. 1995). Microsomal preparations from developing rape seed did not have such phospholipase A_2 activity towards medium chain fatty acids (Stahl et al. 1995).

[0022] If a gene coding for plant phospholipase A_2 with specificities for a particular uncommon fatty acids is expressed in transgenic oil producing organisms engineered to produce that uncommon fatty acids, the recombinant phospholipase A_2 will remove the uncommon fatty acids from the phospholipids of the cell and thereby prevent deleterious effects on cell metabolism caused by the presence of this acid in the membrane lipids. This invention describes how such phospholipase A_2 genes will be isolated and what uses they will have in commercial applications.

[0023] The invention will be described more closely below in association with an experimental part and the enclosed drawings.

[0024] The drawings show:

[0025] Fig.1 shows a SDS-polyacrylamid electrophoresis of soluble developing elm seed PLA_2 purification fractions, followed by colloidal Coomassie staining. Lane A and I contain 100 ng of MW standards (Pharmacia low MW); lane B, 100,000 g supernatant, 50 µg; lane C, ammonium sulphate pellet, 50 µg; lane D, acetone supernatant, 50 µg; lane E, Q Sepharose, 40 µg; lane F, Superose 12, 25 µg; lane G, C_4-HPLC, 2 µg; lane H C_2C_{18}-SMART, 100 µg; and lane J, commercial Naja naja kouthia PLA_2, 100 ng. All samples were reduced with DTT in sample buffer.

[0026] Fig.2 shows PLA_2 activity measurements of gel pieces from whole lanes (5-94 KD) of a SDS-PAGE 8-18% gradient gel. Lane A contains 50 ng of Naja naja kouthia PLA_2 (Sigma) and lane B 50 ng of developing elm seed soluble PLA_2. PLA_2 activity recovered from gel pieces of similar lanes are shown on each side.

[0027] Fig. 3 shows molecular weight data of the purified soluble PLA_2, from MS-Malditof.

[0028] Fig. 4 shows molecular weight data of the purified soluble PLA_2 that has been reduced and iodoacetamide alkylated, from MS-Malditof.

[0029] Fig. 5 shows a SDS-polyacrylamid electrophoresis of purified microsomal peak 11 PLA_2 from developing elm seeds, with recovered PLA_2 activity. Lane A contain about 20 ng of purified peak II PLA_2, non reduced; lane B contain 25 ng of MW standard (Pharmacia low MW). The gel was silver stained.

[0030] Fig. 6 Alignment of deduced amino acid sequence of the full length rice cDNA clone GenBank ID: D49050 with 10 different low molecular weight phospholipase A_2 from animal tissues. Conserved amino acid sequences are boxed. Spaces introduced to optimize alignment are indicated by a dash. The different sequences represent phospholipase A_2 from the following species:

- D00035: *Canis sp.* (SEQ ID NO:1)

- D10070: *Trimeresurusflavolridis* (SEQ ID NO:2)
- M21054: *Homo sapiens* (SEQ ID NO:3)
- X12605: *Notechis scutatus scutatus* (SEQ ID NO:4
- X53406: *Bungarus multicinctus* (SEQ ID NO:5)
- X53471: *Vipera ammodytes* (SEQ ID NO:6)
- X76289: *Bothrops jararacussu* (SEQ ID NO:7)
- Y00120: *Bostaurus* (SEQ ID NO:8)
- Y00377: *Laticauda laticaudata* (SEQ ID NO:9)

[0031] Fig. 7 Alignment of the N-terminal sequence (SEQ ID NO:10) of the purified soluble PLA_2 from elm seeds with deduced amino acid sequences (SEQ ID NOs:11-13) from three EST-clones from rice green shoots, including the cDNA clone D49050 fully sequenced by the inventors. The EST-sequences are denoted by their GenBank accession number. Conserved amino acid positions between the elm and rice proteins as well as the regions with homology to the Ca^{2+}-binding and the active site in animal low molecular weight PLA_2's are boxed. A fourth rice clone (GenBank ID: D47320) with high homology to the three above was found in the EST database, but excluded from the alignment due to lower quality of the DNA sequence.

Experimental Part

[0032] Proteins with phospholipase A_2 (PLA_2) activities towards 1-palmitoyl-2-decanoyl-*sn*-glycerol-3-phosphocholine

were purified from a soluble and microsomal fraction of developing elm seeds according to the following protocols:

Assays of phospholipase A_2 activity.

[0033] Membrane associated PLA_2 activity was assayed according to Stahl et al. (1995) using *sn*-1-palmitoyl-*sn*-2-[^{14}C]decanoyl-*sn*-glycerol-3-phosphocholine as substrate. In standard assays of the solubilized microsomal activity and of the soluble activity 1-palmitoy1-2-[^{12}C]palmitoyl-glycerol-*sn*-3-phosphocholine was used as substrate and was presented as mixed micelles with the non-ionic detergent lubrol PX, in a PC/detergent molar relation of 1:10. Samples, 0.5-10 µl, were assayed for PLA_2 activity by incubation at 30°C for 5-30 min with 5 nmol of ^{14}C-labelled phosphatidylcholine (10,000 dpm/nmol) in a total volume of 50 µl of 50 mM Tris/HCl, pH 8.0 containing 10 mM $CaCl_2$ and 0.06% (w/v) lubrol PX. The reaction was stopped by the addition of 400 µl of chloroform/methanol/acetic acid 50:50:1 followed by 150 µl of H_20. The samples were mixed thoroughly and centrifuged 10,000 g for 1 min. Chloroform phases containing extracted lipids were passed through mini-columns of silica gel, followed by a wash with 400 µl of chloroform. The eluates from the silica columns, containing released [^{14}C]palmitic acid, were collected and analysed by scintillation counting.

[0034] Assays of PLA_2 activity from SDS-gels was performed according to following protocol; A whole or part of a SDS-PAGE (Pharmacia Exelgel 8-18%) lain, not fixed, containing purified PLA_2 were divided in 2-3 mm wide pieces and placed in eppendorf tubes together with 400 µl of 20 mM Tris, pH 8.0 containing SDS 0.5% (w/v). The tubes were rotated end over end at 37° C for 16 h, in order to eluate proteins from the gel pieces. Fractions were concentrated to 100 µl in a Speed-Vac concentrator (Savant) and then precipitated with ethanol/chloroform (Wessel and Flügge 1984) to remove SDS. Air dried pellets were solubilized in 150 µl of 50 mM Tris/HCl, pH 8.0 containing 10 mM $CaCl_2$ and 0.06% (w/v) lubrol PX and activity measurements were started by adding 5 nmol of *sn*-1-palmitoyl-*sn*-2-[^{14}C]palmitoyl-*sn*-glycerol-*sn*-3-phosphocholine (10,000 dpm/nmol) solubilized in 50 µl of 50 mM Tris/HCI, pH 8.0 containing 10 mM $CaCl_2$ and 0.06% (w/v) lubrol PX. The samples were incubated at 30°C for 2-4 h and stopped by adding 400 µl of $CHCl_3$/MeOH/Hac, 50:50:1.

SDS-gel electrophoresis

[0035] Protein fractions were if necessary concentrated in Sped-Vac and precipitated with ethanol/chloroform according to Wessel and Flugge (1984). Samples with a final volume of 20 µl in 50 mM Tris/acetat pH 7.5 with 1% (w/v) SDS, with or without 10 mM of dithiothreitol, were heated to 95°C for 5

min, centrifugated 5 min 13,000 g and loaded on to a horizontal 8-18% gradient polyacrylamid gel (Pharmacia ExelGel SDS) with a 33 mm stacking zone and a 77 mm separating zone. The gel was chromatographed on a Pharmacia Multiphor II unit at 15°C and stained either with colloidal Coomassie (Neuhoff et al 1988) over night or with silver staining

Material

[0036] Developing seeds of elm (*Ulmus glabra*) were harvested and peeled. The white endosperms were immediately frozen in liquid nitrogen and stored in -80°C.

Purification of soluble phospholipase A_2

[0037] 60 g of liquid nitrogen frozen elm endosperm was homogenized with a Ultraturcrax® in 600 ml of ice cold 100 mM potassium phosphate buffer, pH 7.2 containing 0.33 M sucrose. The homogenate was filtered through two layers of Miracloth® and centrifuged 10,000 g for 12 min. The supernatant was filtered through one layer of Miracloth® and centrifuged a second time, at 100,000 g for 90 min. The final supernatant which contained about 80% of the total PLA_2 activity, was brought to 55% (w/v) of ammonium sulphate and left with steering at 4°C for 1 h. Precipitated proteins were pelleted by centrifugation 10,000 g for 10 min and resuspended in 130 ml of 50 mM dietanolamin buffer pH 8.5. Ice cold acetone was added to a final concentration of 45 % (v/v) and the extract

was left at 4°C for 30 min. Precipitated proteins were removed by centrifugation for 10 min at 10,000 g and the resulting supernatant was dialysed against 20 volumes of 20 mM piperidin, pH 11.0, with one change over night. The dialysed extract was applied to a Q-Sepharose Fast Flow 7 ml column (1.0 x 10.0 cm) equilibrated in 20 mM piperidin, pH 11.0. The column was eluted with a linear salt gradient from 100 to 500 mM NaCl in 20 mM piperidin, pH 11.0 at a flow rate of 2 ml/min. 3 ml fractions were collected and assayed for PLA_2 activity. A single broad peak of activity was eluted at a salt concentration of 200 to 300 mM NaCl. Peak fractions were pooled, concentrated on Centricon-10 to 0.6 ml and chromatographed in three separate runs on a Pharmacia Superose 12 (1.0 x 30.0 cm) gel filtration column (0.4 ml/min) in 20 mM Tris/HCI, 150 mM NaCl, pH 8.0. Fractions (0.5 ml) were collected and tested for PLA_2 activity. Peak fractions from all three runs were pooled and the PLA_2 was further purified using a C_4 reversed-phase HPLC column (Vydac 0.46 x 10.0 cm) that was equilibrated in 0.1% trifluoroacetic acid (TFA). The column was developed at 0.4 ml/min with a 30 min gradient (20-45% of acetonitrile in 0. 1% TFA) and peaks monitored at 280 nm were collected manually. Collected fractions from four separate runs were assayed for PLA_2 activity. Peak fractions were pooled and the acetonitrile content was reduced by

evaporation in a Speed-Vac concentrator (Savant). The PLA_2 was finally purified to apparent homogeneity on a C_2C_{18} reversed-phase HPLC column (0.21 x 10.0 cm) equilibrated in 0.1% TFA and developed at 100 µl/min with a 60 min gradient (30-60% acetonitrile in 0.1% TFA) using a SMART system (Pharmacia). Peaks monitored at 280 nm were automatically collected and then subjected to PLA_2 assay. The PLA_2 elutes as a discrete peak in the gradient at about 50% acetonitrile.

[0038] The PLA_2 was purified about 180 000 times from the developing elm seed extract of soluble proteins, to a final specific activity of 44 µmol/min x mg protein (see Table I).

Table I, Purification of Soluble PLA_2 from Developing Elm Seeds

	Protein (mg)	Total Activity (nmol/ml)	Specific Activity (nmol/min x mg protein)	Purification	Yield (%)
100,000 g sup	3,340	833	0.25	1	100
Am. sulphate pell	1,060	563	0.53	2	68
Acetone sup	150	780	5.2	21	94
Q Seph F.F.	24	420	17.5	70	50
Gel Filtration	3.8	263	69.2	277	32
C_4-HPLC	0.09	173	1,922	7,690	21
C_2C_{18}-SMART	0.003	133	44,330	177,300	16

[0039] The final extract showed one major band when subjected to SDS-PAGE on a 8-18% gel and stained with colloidal Coomassie, with a molecular mass of 14 kDa (see Fig 1). Recovered PLA_2 activity from SDS-PAGE gels coincide with the 14 kDa band (see fig. 2). When subjected to Malditof-MS,

the PLA_2 gave two major peaks with the masses 13220 and 13890 and a minor with a molecular mass of 12680 (see fig 3). When alkylated with N-isopropyl all three peaks changed masses with about the same amount, 1150, which would correspond to 12 cysteine residues in each of the three proteins (fig.4).

N-terminal sequence analysis

[0040] About 1 µg of purified PLA_2 was reduced, by incubation in 0.1 M Tris/HCI, pH 8.5 containing 8 M guanidinhydrocloride, 10 mM EDTA and 20 mM DTT at 56° C for 30 min followed by alkylation in 20 mM 4-vinylpyridin for 60 min at room temperature. The reduced and alkylated PLA_2 was desalted, applied on a C_2C_{18} g reversed-phase HPLC column (0.21 x 10.0 cm) equilibrated in 0.1% TFA and eluted at 100 µl/min with a 30 min gradient (30-60% acetonitrile in 0.1% TFA) using a SMART system (Pharmacia). The protein was then subjected to amino-terminal sequence determination by automated Edman degradation using an Aplied Biosystems 476A gase phase protein sequenator. The amino-terminal sequence was manually determined to be:

XNVGVQATGTSISVGKGCF(S)RKCE(P)P(K)F(Y,L)FCYGPXFLR(L)Y(S) (SEQ ID NO:14) (when signals for several amino acids were obtained the minor amino acid signal(s) is denoted in brackets after the main signal). When using the amino-terminal sequence as query for the Basic local alignment search tool at NCBI with

the blastp search program against the Non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR, the tblastn search program against Non-redundant GenBank+EMBL+DDBJ+PDB sequences and Non-redundant Database of GenBank EST Division the best aligned sequences are three EST's (GenBank accession number D47724, D47653 and D47320) derived from green rice shoots. Fig. 7 shows an alignment of the amino-terminal sequence with the deduced amino-acid sequence from two of these EST-clones and the D49050 rice EST-clone. The amino-terminal sequence show significant homology with the rice sequences, notably the positions of the three cysteine-residues are conserved. In addition, predictions of leader peptide cleavage site of rice clones D47724 and D47653 suggest cleavage between G 24 and L 25. This supports the alignment of the amino-terminal sequence to the mature part of the rice sequences Regions with high homology to the conserved Ca^{2+} binding- and active sites of secretory PLA_2's (see fig 6) are both found in all three aligned rice EST's.

Purification of microsomal phospholipase A_2

[0041] 60 g of liquid nitrogen frozen elm endosperm was homogenized with a ultraturrax® in 600 ml of ice cold 100 mM potassium phosphate buffer, pH 7.2 containing 0.33 M sucrose. The homogenate was filtered through two layers of Miracloth® and centrifugated 10,000 g for 12 min. The supernatant was

filtered through one layer of Miracloth® and centrifugated a second time, at 100,000 g for 90 Min. The microsomal pellets were resuspended in 90 ml of 100 mM potasium phosphate, pH 7.2 with a glass homogenizer and, if not used immediately, stored at -80°C. The microsomal membranes were diluted to 150 ml with 100 mM potasium phosphate, pH 7.2 and solubilized by the addition of 150 ml of 200 mM potassium phosphate, pH 7.2 containing glycerol 17% (v/v), lubrol PX 0.6% (w/v) and EGTA 1 mM. The mixture was incubated 15 min at 4°C followed by a centrifugation 100 000 g at 4° C for 90 min. The supernatant was dialysed against two changes of 5 liter of 20 mM dietanolamin, pH 8.5 containing glycerol 8.7% (v/v) and lubrol PX 0.06% (w/v). The dialysed supernatant was passed through a 200 ml Q-Sepharose column (50 x 100 mm) at a flow rate of 3 ml/min. Non-retained material with about 30-50% of the PLA_2 activity was collected and pH adjusted to 5.7 by adding requiring amount of 1 M MES buffer. This fraction was applied to a 7 ml SP-Sepharose column (10 x 100 mm) equilibrated in 50 mM MES, pH 5.7 with glycerol 8.7% (v/v) and lubrol PX 0.06%(w/v) at a flow rate of 3 ml/min. After the sample had passed through, the column was washed with several column volumes of equilibrating buffer and then eluted with a 100 ml linear gradient from 0 to 480 mM of NaCl in the same buffer. Eluated fractions containing PLA_2 activity were pooled and

concentrated to a volume of 200 µl by centrifugation on Centricon-50 and vacuum evaporation in a Speed-Vac concentrator (Savant). The sample was applied to a Superose 12 (10 x 300 mm) Pharmacia column equilibrated in 20 mM Tris, pH 8.0 with glycerol 4.3% (v/v), lubrol PX 0.06% (w/v) and 50 mM NaCl. Fractions with PLA_2 activity were pooled and further purified using a C_4 reversed-phase HPLC column (Vydac 0.46 x 10.0 cm) that was equilibrated in 0.1% trifluoroacetic acid (TFA). The column was developed at 0.4 ml/min with a 30 min gradient (20-45% of acetonitrile in 0.1% TFA) and peaks monitored at 280 nm were collected manually. Collected fractions were assayed for PLA_2 activity, and found to be divided into to activity peaks, one (peak I) which eluted at about 35% acetonitrile and the second (peak II) which eluted at about 47% acetonitrile. Peak fractions were pooled and lubrol PX to a final concentration of 0.5% (w/v) was added before the acetonitrile content was reduced by evaporation in a Speed-Vac concentrator (Savant). The two PLA_2 fractions were both finally purified to near homogeneity on a C_2C_{18} reversed-phase HPLC column (0.21 x 10.0 cm) equilibrated in 0.1% TFA and developed at 30 µl/min with a 60 min gradient (30-60% acetonitrile in 0.1% TFA) using a SMART system (Pharmacia). Peaks monitored at 280 nm were automatically collected and then subjected to PLA_2 assay. The purified peak I PLA_2 gave a

very sharp band on SDS-PAGE 8-18% gradient gel with a molecular mass around 17 KD and the peak II PLA_2 gave a 14 KD band. Both bands coincided with recovered PLA_2 activity from gel pieces. Fig. 5 shows the purified peak 11 PLA_2 separated on a SDS-PAGE followed by silverstaining. After Coomassie staining only the 14 KD band was visible. However, upon silverstaining some minor contaminants show up.

[0042] The microsomal PLA_2 activity was purified from the microsomal fraction with a specific activity of 0.28 nmol/min x mg protein to a specific activity of about 50 µmol/min x mg protein which gives a purification factor of about 100,000.

Properties of purified PLA_2s

[0043] The purified soluble and microsomal PLA_2s have very similar properties. They have a pH optimum between 7 and 9, an absolute requirement for Ca^{2+} for activity with several mM for optimal activity. The activities are extremely stable both to extreme pH values, heat and organic solvents. The activities are, however, sensitive to reducing agents like DTT and mercaptoethanol (see Table II).

Table II: Effects of Reduction, EGTA and Heat on Developing Elm Seed Soluble PLA_2

Treatment	PLA_2 Activity Released [^{14}C]fatty acids (dpm)
Control	4740
05°C for 5 min	5190
Mercaptoethanol 1% (v/v)	170
EGTA 10 mM	170

[0044] The purified PLA_2's hydrolyses the sn-2 position of phospholipids (Table III), and does not show any activity towards diacylplycerols or lysophosphatidylcholine (Table IV).

Table III: Position specificity of soluble developing elm seed PLA_2 assay described above with a partly purified soluble PLA_2 fraction (PC=phosphatidylcholine, LPC=lysophosphatidylcholine)

	Recovered ^{14}C Activity (% of total recovery)		
PC Substrate	fatty acid	PC	LPC
sn-1(16:0-*sn*-2-[^{14}C]16:0	44	56	0.6
di-[^{14}C]16:0	27	50	23

Table IV: Substrate specificity of microsomal PLA_2. Incubations done according to the PLA_2 assay described above (PC=phosphatidylcholine, LPC=*sn*-1-lysophosphatidylcholine, DAG=Diacylglycerol)

	Recovered ^{14}C activity (% of total recovery)			
PC Substrate	fatty acid	PC	LPC	DAG
sn-1-16:0-*sn*-2-[^{14}C]16:0-PC	34	65	0.8	-
di-[^{14}C]16:0-PC	27	40	33	-
sn-1-16:0-*sn*-2-[^{14}C]10:0-PC	44	54	0.6	
[^{14}C]10:0-LPC	0.8	-	98	-
sn-1-16:0-*sn*-2-[^{14}C]10:0-DAG	0.8	-	-	99

[0045] The molecular weight and the biochemical characteristics of both the soluble and microsomal elm PLA_2 suggest that they are related to the well described low MW "secretory" PLA_2s from animal sources. This is further supported by the amino-terminal sequence data and alignments. The secretory PLA_2s have all conserved amino acid sequences at the Ca^{2+} binding site and at the active site as well as cyein residues.

[0046] When searching databases for deposited expressed sequences from plants with homology to low molecular weight

animal phospholipases in Ca^{2+} binding site and active site, the inventors found three anonymous partially sequenced cDNA clones from green shots of (GenBank ID: D49050, D47724, D47653). The cDNA clone D49050 was received upon request from Dr. Yoshiaka Nagamura, DNA Materials Management group, Rice Genome Project, NIAR/STAFF, STAFF Institute, 446-1, Ippaizuka, Kamiyokoba Tsukuba, Ibaraki 305 Japan. The entire cDNA was sequenced and was shown to contain an open reading frame encoding a full length protein of an estimated molecular weight of 15 kDa. An alignment of the deduced amino acid sequence of D49050 with a number of animal low molecular weight PLA_2s is presented in Fig. 6.

[0047] The D49050, D47724, D47653 clones coded for proteins with the same amino acid sequences as in thee Ca^{2+} binding site and active site in the animal low molecular weight PLA_2s and similar to these enzymes they contained several cysteine residues (see Fig. 7). The cDNA clones also coded for amino acid sequences with significant homologies with the N-terminal sequence of the purified phospholipase A_2 from elm seeds where the positions of the three cysteine residues of the elm enzyme was totally conserved in all three cDNAs (see Fig.7). Thus with all probability these rice cDNAs were coding for a plant PLA_2 similar to the enzyme purified from developing elm seeds according to the invention.

[0048] By expressing this cDNA in suitable organism, like bacteria, for example *E. coli*, yeast or plants, a recombinant PLA_2 protein will be obtained and PLA_2 activities can be demonstrated. Although the physiological function of the rice enzyme is unknown, a function in rice shoots could be removal of oxygenated fatty acids from membrane lipids, as has been shown to take place in e.g. wheat roots (Banes et al, 1992).

[0049] By constructing degenerated nucleotide primers based on suitable amino acid sequences of the soluble elm PLA_2 and rice cDNA clones amplification of elm fragments containing the corresponding sequences will be done from cDNA or genomic DNA from elm seeds by PCR. These fragments will be used as probes for screening for the elm cDNA PLA_2 from a cDNA library from developing elm seeds.

[0050] Since phospholipid acyl hydrolases with high specificities towards epoxy and hydroxy fatty acids have been described in membrane preparations from other plant species (Stahl et al. 1995) homologous cDNA coding for PLA_2 with other acyl specificities than the elm enzyme can be isolated from other plant species with the aid of the cDNA encoding for the elm PLA_2 and/or the rice cDNA clones as probes. Alternatively suitable amino acid sequences of these enzymes can be used to construct degenerated nucleotide primers and amplify cDNA fragments derived from the other plant species. These

fragments will be used as probes for screening for the cDNA coding for PLA_2 from a cDNA library from other plant species.

[0051] When a cDNA clone containing a full length cDNA or genomic DNA coding for a PLA_2 have been obtained this cDNA can be used for transformation.

[0052] According to the invention, the PLA_2 gene, i.e. the PLA_2 cDNA or genomic clone, is used in combination with a gene for an uncommon fatty acid for obtaining transgenic plants comprising both said genes. The transgenic plants are obtained by using said plant phospholipid acyl hydrolase gene for transforming transgenic oil accumulating organisms engineered to produce said uncommon fatty acid. Alternatively, transgenic plants are obtained by using the plant phospholipid acyl hydrolase gene for transforming oil accumulating organisms, which are crossed with other oil accumulating organisms engineered to produce said uncommon fatty acid.

REFERENCES

Badami, R.C., and Patil, K.B. (1981). Structure and occurrence of unusual fatty acids in minor seed oils. Progress in Lipid Research, 19, 119-53.

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. & Stymne, S (1990) Regulation of triacylplycerol biosynthesis in embryos and microsomal fractions from the developing seeds of Cuphea lanceolata. Biochem. J. 272, 31-38

Bafor, M., Srnitl;l, M.A., Jonsson, L., Stobart, K. & Stymne, S. (1993) Biosynthesis of vernoleate (cis-12-epoxyoctadeca-cis-9-enoate) in microsomal preparations from developing endosperm of Euphorbia lagascae. Arch. Biochem. Biophys. 303, 145-151

Banas, A., Johansson, I. & Stymne, S. (1992) Plant microsomal phospholipases exhibit preference for phosphatidylcholine with oxygenated acyl groups. Plant Science 84, 137-144

Kohn, G., Hartmann, E., Stymne, S. & Beutelmann, P. (1994) Biosynthesis of acetylenic acids in the moss Ceratodon purpureus. J. Plant Physiol. 144, 265-271

Neuhoff, V., Arold, N., Taube, D. and Ehrhart, W. (1988) Improved staining in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9, 255-262

Stymne, S (1993a) Biosynthesis of storage fat in oil crops - today and tomorrow. In: Phytochemistry and Agriculture (Eds. T.A. van Beek & H. Breteler. pp. 288-312. Clarendon Press, Oxford)

Stymne, S. (1993b) Biosynthesis of uncommon fatty acids and their incorporation into triacylglycerols. In: Biochemistry and Molecular Biology of membrane and Storage Lipids of Plants (eds. N. Murata & C. Somerville), pp. 150-158. American Society of Plant Physiology, Rockville.

Stymne, S., Bafor, M., Jonsson, L., Wiberg, E., and Stobart, A.K. (1990) Triacylglycerol assembly. In Plant Lipid Biochemistry, Structure and Utilization, (eds. P.J. Quinn and J.L. Harwood), pp. 191-97. Portland Press, London

Stahl, U., Banas, A. & Stymne, S. (1995) Plant microsomal phospholipid acyl hydrolases have selectivities for uncommon fatty acids. Plant Physiol. 107, 953-962

Voge!, G. & Browse, J. (1995) Role of choline phosphotransferase and diacylglycerol acyltransferase in channelling unusual fatty acids into the triacylglycerol pool during oil seed development. In: Plant lipid metabolism (eds. J-C. Kader & P. Mazliak), pp. 506-508. Klower Academic Press, Dordrecht

Wessel, D. and Flugge, U. I. (1984) A method for quantitative recovery of protein in diluted solution in the presence of detergents and lipids. Biochemistry 138, 141-143

Wiberg, E., Banas, A. & Stymne, S. (1995) Partitioning of medium chain fatty acids between membrane and storage lipids in laureate producing rape. Abstract 039 in abstract book from the symposium: Biochemistry and Molecular Biology of Plant Fatty acid and Glycerolipid. June 1-4, South Lake Tahoe, California